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Plasticity of thermal tolerance and metabolism but not water loss in an invasive reed frog

Sarah J. Davies [a,*], Melodie A. McGeoch [b], Susana Clusella-Trullas [a]

[a] DST-NRF Centre of Excellence for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa
[b] School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia

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ABSTRACT

Phenotypic plasticity may buffer the selection pressures on organisms that inhabit novel or rapidly-changing environments. We investigated plasticity of thermal tolerance, energetic and water loss traits and their interaction with behaviour in a small-bodied, arboreal anuran (*Hyperolius marmoratus* Rapp, Hyperoliidae) undergoing rapid range expansion into the winter rainfall region of South Africa. After short-term exposure to three temperatures (acclimation treatments) commonly encountered in their historical and novel ranges, frogs exhibited a broad thermal tolerance range (mean ± s.d.: 42.1 ± 2.9 °C) and higher plasticity in CT_{max} than in CT_{min}. Resting metabolic rate was lowest in cold-acclimated animals, while active metabolic rates were lowest in warm-acclimated frogs, likely reflecting compensation towards energy conservation. Evaporative water loss was not significantly altered by the acclimation treatments in either resting or active animals, indicating limited plasticity in this trait compared to metabolism. Our results suggest that plasticity of temperature limits and metabolism may benefit this species in variable environments such as those encountered in its expanded range. Lack of plasticity in water loss during resting and activity suggests that these frogs rely on their high cutaneous resistance and behavioural means to buffer climate variation. This study highlights the importance of synergistic interactions between physiology and behaviour in determining amphibian responses to temperature variation.

1. Introduction

The capacity of organisms to respond to environmental change through morphological, physiological and behavioural plasticity is likely to contribute to mitigating, at least to a point, the negative effects of habitat loss, climate change, biological invasions and epidemics (e.g. Agrawal, 2001; Cooke et al., 2013; Peacor et al., 2006; Seebacher and Franklin, 2012). Determination of the magnitude and direction of phenotypic plasticity in multiple physiological traits aids the development of mechanistic models for improved prediction of organism responses to environmental change (Basson and Clusella-Trullas, 2015; Buckley et al., 2015; Winwood-Smith et al., 2015). Studies investigating reversible plasticity of multiple thermal traits in anurans are uncommon, and represent only a minority of taxa and functional groups (see Lotshaw, 1977 for *Rana* spp.; Carey, 1979 for *Bufo boreas*; Rogers et al., 2007 for *Limnodynastes peronii*; Overgaard et al., 2012 and Seebacher and Franklin, 2011 for *Rhinella marina*). Arboreal frogs face particular thermal and hydric challenges, as they typically travel long distances and spend significant lengths of time away from surface water. Body size influences time to desiccation (Tracy et al., 2010; Tracy et al., 2013), and for small-bodied arboreal frogs, the ability to avoid or delay desiccation stress is pivotal to survival. Quantifying the level of plasticity in traits of environmental stress resistance for these organisms would therefore be essential for improving predictions of their future distribution and potential vulnerability to environmental change.

The painted reed frog (*Hyperolius marmoratus* Rapp; body mass 1–3 g) has a broad distribution in southern African savannas (Bishop, 2004; Channing, 2001; Fig. A1) and has established breeding populations in the south-western Cape of South Africa since the late 1990s (Bishop, 2004; Davies et al., 2013; Tolley et al., 2008). The novel range is characterised by a more variable, temperate, winter-rainfall Mediterranean climate and dry, hot summers, in contrast with the largely subtropical, summer-rainfall historical range (Tyson, 1986). The breeding phenology (timing of reproduction) of the painted reed frog has remained unchanged in the novel range and the peak activity period coincides with the hottest, driest period of the year (SJD, unpublished data) instead of the warm but humid conditions found in its original geographic range. Correlative models indicate that the cold winters and warm, dry summers typical of the novel range may limit the frogs' ability to occupy further habitat (Davies et al., 2013). However, correlative relationships do not necessarily reveal trait-environment relationships that cause range limits or niche shifts (Buckley et al., 2010; Kearney and Porter, 2004). The magnitude and extent of phenotypic

* Corresponding author at: Centre for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa. Tel.: +27 21 808 3922; fax: +27 21 808 2995.
E-mail address: sdavies@sun.ac.za (S.J. Davies).

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plasticity of performance in response to temperature variation of painted reed frog populations in the novel range may help clarify the capacity for this species to persist in these new conditions.

While most anurans lose water at a rate similar to that of a free water surface (Spotila and Berman, 1976), painted reed frogs are highly desiccation-resistant (Geise and Linsenmair, 1988; Withers et al., 1982). The mechanisms of this resistance are unknown, but may involve the structure and composition of the dorsal skin (Kobelt and Linsenmair, 1986; Lillywhite, 2006). In addition, resting frogs adopt a water-conserving posture with limbs tucked under the body and head lowered to be in contact with the substrate. During activity, exposure of the highly permeable ventral and axial skin is associated with high water loss rates (Geise and Linsenmair, 1986; Kobelt and Linsenmair, 1986). Painted reed frogs are prolonged breeders whose period and manner of calling is energetically costly (Bishop, 2004; Wells, 2007). The ability to attend a chorus and call for several consecutive nights increases male mating success, whereas body size and physical condition appear to play secondary roles (Dyson et al., 1998; see also Llusia et al., 2013; Rogers et al., 2007).

This study aimed to assess the thermal acclimation (i.e. phenotypic plasticity) of three key physiological traits: critical thermal limits (measured by CT_{min} and CT_{max}), metabolism (resting and active metabolic rates; RMR and AMR, respectively) and water loss (resting and active water loss rates, WLR). First, we predict that CT_{min} and CT_{max} will respond to acclimation temperature in a way that favours the organism in a variable environment. Second, because metabolic rate represents an energetic cost of living (Hulbert and Else, 2004), we hypothesise that lower RMR is beneficial, with the assumption that additional energetic resources can be allocated to capacity functions such as movement to and from nocturnal choruses, egg production, calling and combat (Boratyński and Koteja, 2010; Burton et al., 2011; Rogers et al., 2007). Therefore, RMR should stay constant or decrease in response to increasing acclimation temperature. Alternatively, RMR may be expected to increase under these conditions if a change in body composition (e.g. muscle mass, organ size) to facilitate movement is associated with increased RMR (reviewed in Piersma and van Gils, 2011). We test this hypothesis by evaluating explicit predictions about the acclimation responses of RMR within a rigorous experimental framework. We distinguish among five competing hypotheses: beneficial acclimation, 'hotter is better', 'colder is better', optimal acclimation temperature and no acclimation response (after Clusella-Trullas et al., 2010; Deere and Chown, 2006; Huey et al., 1999; Kingsolver and Huey, 2008; Marais and Chown, 2008). Predictions of the shape and direction of the expected relationships for metabolic rates are shown in Table 1. Third, we expect AMR to be up-regulated in response to cold acclimation treatments, thereby reflecting the upregulation of biochemical processes for function in the cold, so that animals can undertake the activities required for reproduction, foraging and dispersal during adverse cold conditions. Fourth, due to the known high desiccation resistance of resting frogs and typical water-conserving posture in this species (Geise and Linsenmair, 1988; Withers et al., 1982), we predicted that resting WLR would remain low and constant over a range of acclimation and test temperatures, while active WLR should perhaps demonstrate compensatory responses that minimise excessive water loss during activity (i.e. similar or lower active WLR at high acclimation temperature compared to the acclimation reflecting mean conditions).

Table 1
Hypothesised significance of the polynomial components of treatment effects (acclimation (ACC) and test temperature (TT)) on resting metabolic rate and water loss rate in painted reed frogs, assuming that lower RMR and resting WLR enhance fitness via energy and water savings. Derived from Huey et al. (1999), Deere and Chown (2006), Marais and Chown (2008), Kingsolver and Huey (2008), and Clusella-Trullas et al. (2010).

Hypothesis	ACC	TT	Interaction (ACC × TT)
Beneficial acclimation (BAH)			L+/−
Colder is better (CIB)	L+	L+	ns
Hotter is better (HIB)	L−	L−	ns
Optimal acclimation temperature (OAH)	Q+		
No acclimation response	ns	ns	ns

L denotes a linear and Q a quadratic component; +/− denotes the sign of the component; ns = relationship not significant; blank = no prediction. Because low resting metabolic rate is assumed to be beneficial (see text), CIB is indicated by a significant positive trend, and HIB by a negative trend in ACC and TT; BAH would be supported by a significant interaction between ACC and TT and OAH by a quadratic effect of ACC; non-significant trends throughout indicate no trait plasticity; blank cells indicate no particular hypothesis about the relationship.

2. Materials and methods

2.1. Collections and acclimation treatments

Adult frogs were collected from three artificial water bodies (dams) in the novel range in the Western Cape Province, South Africa (Durbanville: 33.872°S 18.624°E, Stellenbosch: 33.992°S 18.820°E, Strand: 34.098°S 18.821°E) during the peak reproductive activity period (austral summer). These collection sites were selected in order to represent the genetic structure that exists across the novel range populations of this species (Tolley et al., 2008). All collection sites were more than 350 km from the historical range and the inter-site distance averaged 21.4 km. Male and female frogs were captured at night, placed in clear plastic bags and transported to the laboratory where they were transferred to glass aquaria (200 W × 500 L × 350 H mm) within 24 h. Aquaria were assigned to one of three temperature treatments (each treatment contained four aquaria) such that male and female frogs from each collection site were represented equally in each treatment. The temperature treatments are referred to as 'acclimations' hereafter. Aquaria were maintained inside temperature-controlled chambers (LTIE 400 and LTGC 200; LabCon, Maraisburg, South Africa) on a controlled 12 h:12 h L:D cycle for at least 14 days prior to each experiment. We used this intermediate photo-period because the time in captivity was relatively long (mean = 114 d, min. = 105, max. = 131 d) given the multiple trials we conducted; we wished to maintain a constant photo-period throughout the study as the focus was to determine the response to temperature acclimation principally, and not day length or an interaction between the two.

The temperature inside each aquarium was monitored using iButton temperature loggers (Thermochron; Dallas Semiconductor, Sunnyvale, CA, USA; www.maxim-ic.com). Aquaria contained 9–11 frogs of mixed sexes and sites of origin, *c.* 3 l of water, plants for perches (*Polyganum* sp., *Cyperus* sp., *Papyrus* sp.) and a small area of soil, and were sprayed with dechlorinated water several times daily to maintain high humidity levels. Frogs were fed *ad libitum* on captive-bred Mediterranean fruit flies (*Ceratitis capitata*), based on observed feeding rates (5–8 flies per individual per day). Prior to acclimation, frogs were uniquely marked with Visible Implant Alpha tags (1.2 mm × 2.7 mm) inserted under the skin of an upper hind limb (Northwest Marine Technology Inc., Shaw Island, WA, USA) and the incision sealed with tissue glue (Histoacryl; Braun, Melsungen, Germany). Non-gravid females were used as far as gravidity could be ascertained from external examination. During the peak breeding season, a proportion of the females collected (±20%) were unavoidably gravid. These females were not excluded because of the difficulty of finding sufficient females at breeding sites on a given night, and because we wanted to assess the effects of sex on the physiological traits.

Air temperatures were recorded in exposed calling and perching microsites at each of the collection sites for a full year prior to collections. Acclimations (15, 20 and 25 °C) were within the range of conditions encountered at field sites (for details see Table 2). The lowest acclimation treatment of 15 °C did not trigger a dormancy state or torpor and frogs rapidly became active when disturbed (see also Schmuck and Linsenmair, 1997). Fifteen degrees Celsius is a common temperature even in summer in the novel range and painted reed frogs are heard calling at temperatures as low as 12 °C (SJD, pers.

Table 2
Environmental temperatures (monthly mean, minimum and maximum) measured at the collection sites. Temperature data (°C) was recorded hourly from October 2008 to November 2009, 1 year prior to collection of the first experimental animals, at semi-exposed calling sites ± 1 m above water level among fringing vegetation (shaded iButton Hygrochron temperature and humidity loggers; Dallas Semiconductor, Sunnyvale, CA, USA; www.maxim-ic.com). Values in the header row are mean CT_{min} and CT_{max} for frogs from each site, averaged across all acclimations. See Fig. A1 for locations of sites.

Month	Durbanville (CT_{min} 2.3 °C, CT_{max} 44.5 °C)			Stellenbosch (CT_{min}1.7 °C, CT_{max} 43.9 °C)			Strand (CT_{min} 2.2 °C, CT_{max} 44.1 °C)		
	Average	Min.	Max.	Average	Min.	Max.	Average	Min.	Max.
Nov 08	17.5	9.5	34.3	18.2	6.7	34.1	18.6	5.8	39.2
Dec 08	19.4	13.4	31.1	20.8	8.3	37.7	21.2	7.7	39.1
Jan 09	20.1	10.3	38.0	21.0	9.9	33.9	22.2	9.3	40.7
Feb 09	22.8	9.9	43.6	22.3	9.7	38.5	23.8	9.8	46.1
Mar 09	21.4	9.9	46.8	20.4	8.4	43.8	20.8	6.6	47.1
Apr 09	17.6	9.5	38.0	17.5	8.1	39.7	16.3	8.6	31.3
May 09	14.5	7.1	33.3	14.4	6.8	31.3	13.1	6.7	22.6
Jun 09	13.1	3.8	30.4	12.8	3.8	30.5	12.0	4.6	22.0
Jul 09	12.7	2.7	27.5	12.4	2.1	29.6	12.0	4.0	24.6
Aug 09	12.8	3.3	32.2	12.8	3.4	29.2	12.2	5.1	24.8
Sep 09	13.9	2.9	35.9	14.0	2.8	31.9	13.3	3.7	24.3
Oct 09	17.2	5.8	36.9	17.2	6.3	33.4	16.7	7.1	28.7

obs.). Critical thermal limits, metabolic and water loss rates were measured on two samples of frogs collected in 2010/2011 (referred to as 'year 1') and 2011/2012 ('year 2'). In year 1, resting metabolic rate (RMR) and resting water loss rate (resting WLR) were measured first, followed by CT_{min} and then CT_{max}. In year 2, CT_{min} and CT_{max} were measured first followed by active metabolic rate (AMR) and active WLR. Post-prandial metabolic rate responses of anurans tend to dissipate within two days, and smaller body size generates a shorter time to peak (Secor, 2009). Therefore, frogs were fasted for 48 h prior to experiments to minimise the effects of specific dynamic action on the experimental results. Animals were blotted with paper towel and weighed before and after each experiment (Toledo AX504, 0.1 mg precision; Mettler, Columbus, OH, USA).

2.2. Human and animal rights

The work was carried out under permits from Western Cape Nature Conservation Board (permit numbers 0035-AAA004006-00206; 0035-AAA004-01054) and Stellenbosch University's Research Ethics Committee for Animal Care and Use (permit number 10NP-DAV01).

2.3. Critical thermal limits

Critical thermal limits were determined on 65 frogs in year 1 (15 females, 50 males) and 63 (30 females, 33 males) frogs in year 2, using the dynamic temperature ramp method (Lutterschmidt and Hutchison, 1997). Frogs were placed individually in cylindrical chambers (37 mm W × 48 mm H) embedded in a fluid-filled Perspex jacket and connected to a programmable water bath (Grant Gr150; Grant Instruments, Shepreth, UK) containing a 1:1 water–glycol mixture. Chamber apertures were closed with acetate film to prevent escape and to maintain the targeted temperature and high humidity (~100% RH) within the chambers. Pilot trials showed that frogs' core temperature equilibrated quickly with the chamber temperature. Two to three frogs were tested at a time and temperatures of four experimental chambers (including an empty chamber) were recorded for the duration of experiments using thermocouples (Type T, 40 gauge) and connected to a data logger (Pico TC-08; Pico Technology, Cambridgeshire, UK). Frogs were allowed to equilibrate in the chambers for 10 min at 20 °C prior to temperature ramping down (CT_{min}) or up (CT_{max}) at a constant rate (0.5 ± 0.02 °C min^{-1} and 0.4 ± 0.05 °C min^{-1}, respectively).

For CT_{min}, frogs were first observed at 20, 15, 10, 9, 8 and 7 °C and then turned over their backs every 1 °C and from 3 °C, every 0.5 °C, to check their righting response (Lutterschmidt and Hutchison, 1997). CT_{min} was reached when a frog could not right itself. For CT_{max}, frogs were observed at 20, 25, 30 and 35 °C and then checked every 1 °C. CT_{max} was recorded when frogs were unable to adhere to the chamber wall, fell on their backs and were unable to right themselves. After all trials, the frogs were placed at room temperature to recover for 10 min before being weighed (all individuals fully recovered). Critical thermal limit trials were performed ~50 days after RMR and resting WLR experiments and frogs were allowed to recover for ~16 days in between CT_{min} and CT_{max} trials. Frogs were maintained at their acclimation temperatures between experiments. All frogs survived critical thermal limit experiments, recovering within 3–5 min. of removal from the experimental chamber.

2.4. Resting metabolic rate and water loss rate

Measurements were made on 105 frogs (13 females, 92 males) from the same collection made for year 1 critical thermal limit experiments. Experiments were conducted during the natural resting period (08 h00–18 h00, Telford and Dyson, 1988), at test temperatures of 15, 20 and 25 °C; lights were kept on throughout the experiments. Eight to ten individuals were assayed at a single randomly-selected test temperature on each day; each individual was tested at a single temperature in a randomised block design. Flow-through respirometry was conducted using a calibrated LI7000 infrared CO_2 and H_2O analyser (LiCor, Lincoln, NE, USA). VCO_2 was measured in preference to VO_2 because of the greater sensitivity of VCO_2 given the small body size of this frog. The gas analyser, together with the glass cuvette (12.5 ml) and activity detector (AD-1; Sable Systems International, Las Vegas, NV, USA) were placed inside a temperature-controlled chamber. Dry, CO_2-free compressed air (20.9% O_2, balance N_2) was scrubbed with soda lime, silica gel and Drierite (Xenia, OH, USA) and flushed at 100 ml min^{-1} through the cuvette (system response time: 35 s for 99% exchange; Lighton, 2008) using a two-channel mass flow controller (Sierra Instruments, Monterey, CA, USA). Baseline readings were taken for 10 min before and after each recording to correct for analyser drift. Temperature inside the cuvette was monitored using thermocouples connected to a Pico TC-08 data logger. Each frog was allowed to equilibrate to the targeted cuvette temperature for 10 min prior to 45 min recordings and was weighed before and after each measurement. Frogs settled within 1 min in the small cuvette and stayed immobile against the curved glass surface.

2.5. Active metabolic rate and water loss rate

Experiments were conducted during the main activity period of the frogs (18 h00–23 h00, Telford and Dyson, 1988), using an experimental setup similar to that described for RMR above, but with a flow rate of 200 ml min^{-1} and a 53 ml glass cuvette (response time: 73 s); lights were kept off inside the temperature-controlled chamber simulating activity periods. Animals were allowed to equilibrate and rest inside the cuvette for 10 min. Thereafter, the cuvette was rotated at a constant rate by a motor (approx. 15 rpm, 0.023 m s^{-1}, Fig. A2). Frogs had to move continuously to maintain an upright position and were prevented from clinging to the side walls of the cuvette by two v-shaped wires placed inside the chamber and controlled by an external magnet on a camshaft. This level of activity was assumed to be higher than voluntary activity and lower than critical activity levels (O'Steen and Bennett, 2003; Walsberg, 1986), and to relate to dispersal or sustained locomotion (rather than capacity). In pilot trials, animals became exhausted after about 10–12 min., so activity tests were limited to 10 min and monitored with an infra-red webcam (Genius eFace 1325R; Genius, Taipei, Taiwan). Ten male frogs from each acclimation treatment (15 °C, 20 °C and 25 °C) were tested at 15 °C, 25 °C and 35 °C in a full factorial design (n = 30). Frogs were subjected to test temperatures in

random order and separated by a recovery period of at least seven days (mean ± s.d.: 9 ± 3 days).

2.6. Data analysis

All analyses were conducted in R version 2.14.0 (R Development Core Team, 2010). Body mass, metabolic rate and water loss rate were $\log_{10}$ transformed to improve the normality of the data. Unless otherwise stated, all means are expressed ± s.d.; pairwise tests are Wilcoxon rank sum tests with Bonferroni correction for multiple tests. Selected interaction terms of interest were included in initial models and model reduction was carried out using likelihood ratio tests and Akaike weights (Anderson and Burnham, 2002). Parameter strengths were assessed using the Car package in R (Fox and Weisberg, 2011). Effect sizes (plasticity) were expressed as the mean of differences between the mean trait values for each acclimation group and the overall mean trait value. Correlations between traits were examined using non-parametric Spearman's rank correlation coefficients (*rho*).

Analysis of covariance (ANCOVA) was used to test for the effect of acclimation (ordered categorical variable) and body mass measured before the experiment (continuous covariate) on critical thermal limits. Because sites are known to have different invasion histories, and introductions are ongoing (Davies et al., 2013; Tolley et al., 2008), collection site was included as a categorical covariate.

The mean body mass of sampled frogs was 1.3 g (initial mass after collection). In both sampling years, frogs from Durbanville were largest. In year 1 they were significantly larger than Strand frogs ($z = -2.810$, $p = 0.005$) but not significantly different from Stellenbosch frogs ($z = -0.311$, $p = 0.755$); in year 2 Durbanville frogs were significantly larger than Stellenbosch frogs ($z = -2.431$, $p = 0.015$) but not those from Strand ($z = -1.579$, $p = 0.114$). Sex was included as a covariate in models to determine whether there was a mass-independent effect of sex. Females were significantly larger than males (females: 1.6 ± 0.4 g, n = 46; males: 1.2 ± 0.3, n = 150; W = 2925.5, $p < 0.001$, across all animals collected). Males were larger in the second year than in the first year (year 1: 1.3 ± 0.2 g, year 2: 1.4 ± 0.3 g, W = 544, $p = 0.009$) but no differences in mass were found for females (year 1: 1.5 ± 0.3 g, year 2: 1.7 ± 0.3 g, W = 159, $p = 0.116$). Therefore critical thermal limit data collected in years 1 and 2 were analysed separately. There were no significant differences in body mass among acclimations (15 °C: 1.4 ± 0.3 g, 20 °C: 1.4 ± 0.3 g, 25 °C: 1.4 ± 0.2 g, $z \leq 0$, $p > 0.1$ for all comparisons).

Respirometry data were processed in Expedata ver. 1.0.24 (Sable Systems International, Las Vegas, NV, USA). For resting trials, the portion of each trace with the lowest CO_2 in ppm and H_2O in ppt corresponding to a resting state from the activity detector trace was selected (20 ± 8 min, n = 105) and transformed to ml CO_2 h^{-1} and mg H_2O h^{-1} using standard equations (Lighton, 2008). ANCOVA was used to test for differences among acclimations, test temperatures, sex and collection site, while adjusting for body mass, on resting metabolic and water loss rate. We used ordered factors ANOVA with orthogonal polynomial contrasts to identify the specific form and direction of the acclimation response for RMR and resting WLR (Clusella-Trullas et al., 2010; Huey et al., 1999). Explicit predictions for the direction and shape of acclimation responses of RMR, resting WLR and AMR were tested among five competing hypotheses (Clusella-Trullas et al., 2010; Deere and Chown, 2006; Huey et al., 1999; Kingsolver and Huey, 2008; Marais and Chown, 2008). Briefly, the 'beneficial acclimation' hypothesis predicts that individuals acclimated to a particular environment (in this case an acclimation temperature) will perform better in that environment than individuals acclimated to other environments. In contrast, the optimal acclimation temperature hypothesis predicts that animals acclimated at intermediate temperatures will perform better at that temperature than individuals acclimated at higher or lower (i.e. more extreme) temperatures. 'Hotter is better' and 'colder is better' describe the situations in which individuals acclimated to warmer or cooler temperatures, respectively, have enhanced fitness compared to individuals exposed to other temperatures. 'No acclimation response' predicts no plasticity in the focal trait. These hypotheses lead to the expected relationships shown in Table 1. Orthogonal contrasts were formulated with the residuals of the metabolic rate-body mass relationship as the response variable, and acclimation (15 °C < 20 °C < 25 °C) and test temperature (15 °C < 20 °C < 25 °C) as ordered factors (Crawley, 2007). Sexes were pooled for this analysis. Within-group sample sizes (10 to 13 individuals per acclimation × test temperature group) closely approximated a balanced design.

Because each individual frog was subject to repeated measurements of AMR at different test temperatures, linear mixed-effects models were used with acclimation temperature, test temperature, site and body mass as fixed effects and individual as a random effect. The addition of the random effect significantly improved model fit (Bates, 2010; Zuur et al., 2008). However, mixed modelling was inappropriate for active WLR data because the addition of the random effect to the model did not significantly improve model fit, so a generalised linear model with Gaussian error structure and identity link function was used to model active WLR.

3. Results

3.1. Microsite temperatures at breeding sites

Mean monthly minimum and maximum temperatures were not significantly different across collection sites during the year preceding the first year of sampling (mean = 6.8 °C, mixed effects model, $t = -2.032$, $p = 0.054$ and mean = 34.2 °C, $t = -1.922$, $p > 0.068$ for mean monthly min and max, respectively) during which study animals likely metamorphosed.

3.2. Critical thermal limits

CT_{min} ranged from 1.2 ± 1.4 °C to 3.1 ± 1.0 °C depending on the acclimation treatment applied (Table 3). Pairwise tests showed that CT_{min} was significantly higher in frogs acclimated at 25 °C than in those acclimated at 20 °C in both years (year 1: 2.1 ± 0.8 vs. 1.3 ± 0.9 °C, $p = 0.002$; year 2: 3.1 ± 1.0 vs. 2.2 ± 0.8 °C, $p = 0.011$) and significantly higher than in frogs acclimated at 15 °C in year 1 (2.1 ± 0.8 vs. 1.2 ± 1.4 °C, $p = 0.012$; Fig. 1a, b; Tables 3; 4). The average effect size of acclimation temperature on CT_{min} was 0.3 °C. In all acclimation groups, CT_{min} was lower in year 1 than in year 2 (Table 3). In year 1, frogs from Durbanville had higher CT_{min} than those from Stellenbosch, with Strand being intermediate between the others (Fig. 1a, b; Table 4; Table A1). There were no consistent inter-site differences in year 2. The linear relationship between CT_{min} and body mass showed that CT_{min} increased with body mass in 15 °C- and 20 °C-acclimated frogs ($F_{1,39} = 14.200$,

Table 3
Comparison of mean values of CT_{min} and CT_{max} (°C) of painted reed frogs tested in each year of the study (year 1—2010/2011; year 2—2011/2012). Data are mean ± s.d. Bold text indicates significant differences between years (alpha = 0.05).

ACC	Year 1		Year 2		Test statistic (W)	*p* value
	Mean ± s.d.	n	Mean ± s.d.	n		
CT_{min}						
15	**1.2 ± 1.4 [a]**	**20**	**2.8 ± 1.5 [c, d]**	**21**	**89.5**	**0.002**
20	**1.3 ± 0.9 [a]**	**23**	**2.2 ± 0.8 [c]**	**21**	**84.5**	**<0.001**
25	**2.1 ± 0.8 [b]**	**22**	**3.1 ± 1.0 [d]**	**21**	**107.5**	**0.003**
CT_{max}						
15	**43.4 ± 2.1 [e]**	**20**	**41.2 ± 2.7 [g]**	**21**	**313.5**	**0.007**
20	44.5 ± 2.0 [e, f]	23	44.3 ± 2.5 [h]	21	268.5	0.533
25	45.4 ± 1.6 [f]	22	46.4 ± 2.0 [i]	21	151.0	0.053

Different superscripts indicate significantly different pairs of group means ($p < 0.05$). All pairwise comparisons are Wilcoxon rank sum test with Bonferroni correction.

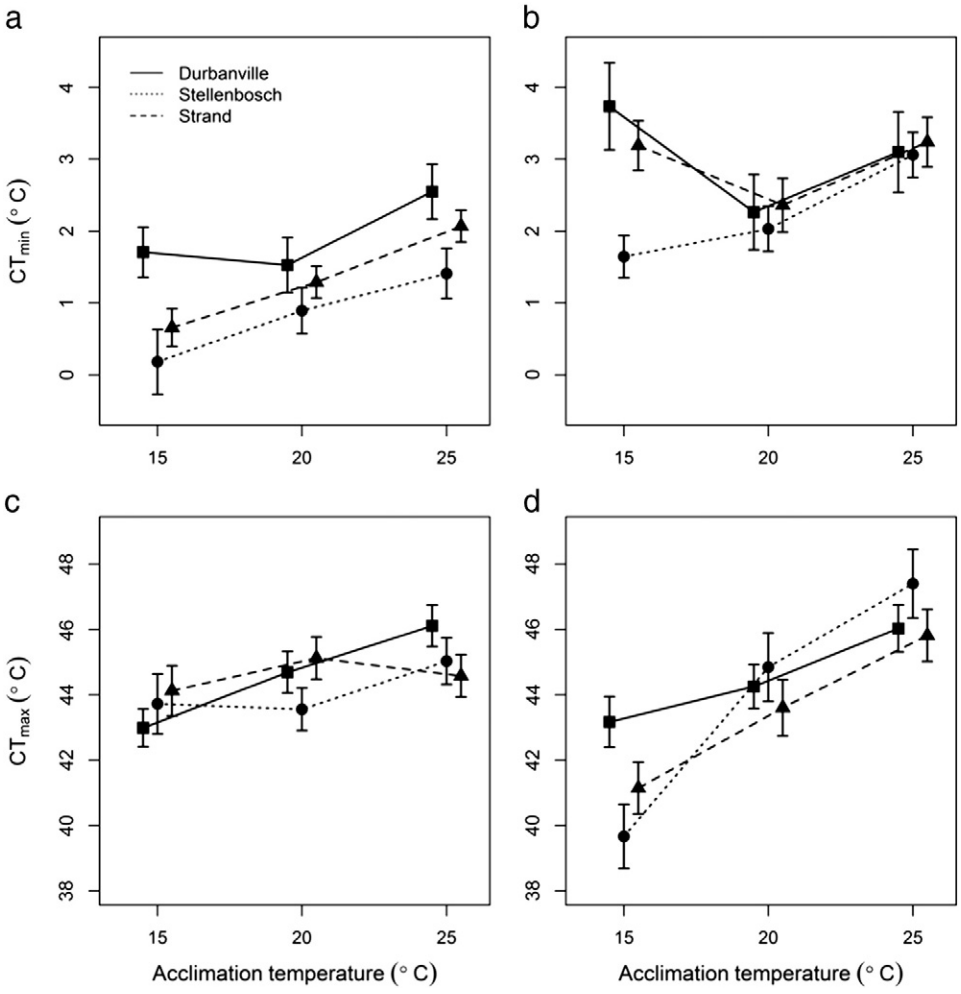

Fig. 1. Critical thermal minima (CT_{min}) and maxima (CT_{max}) of painted reed frogs from three sites in the novel range tested in two different years. Panels a and c: year 1 (2010/2011), panels b and d: year 2 (2011/2012). Data are means ± s.e.m.

$p < 0.001$, $F_{1,42} = 9.879$, $p = 0.003$, respectively) but did not change with body mass in 25 °C-acclimated frogs ($F_{1,41} = 0.053$, $p = 0.819$).

CT_{max} increased with acclimation temperature, but this effect differed between years (acclimation × year interaction, Table 4; Table A1). The average effect size of acclimation on CT_{max} was 1.3 °C and group means ranged from 41.2 ± 2.7 °C to 46.4 ± 2.0 °C depending on acclimation temperature and sampling year (Table 3). In year 1, 25 °C-acclimated frogs had significantly higher CT_{max} than those acclimated at 15 °C, but not 20 °C (Fig. 1c); in year 2, CT_{max} increased significantly with acclimation temperature (Fig. 1d; Table 3). Frogs acclimated at 15 °C had higher CT_{max} in year 1 than in year 2, but this was not true for the other acclimation groups (43.4 ± 2.1 vs. 41.2 ± 2.7 °C, $p = 0.007$; Fig. 1c, d; Table 3; Table A1). Body mass had a negative effect on CT_{max} in all acclimation groups, but was only significant in 20 °C-acclimated frogs ($F_{1,42} = 7.119$, $p = 0.011$).

The mean thermal tolerance range (calculated from absolute differences between CT_{min} and CT_{max}) across all acclimations was 42.1 ± 2.9 °C (min. = 32.4 °C, max. = 48.0 °C). Thermal tolerance range increased with acclimation temperature ($F_{1,120} = 14.280$, $p < 0.001$) with an average effect size of 1.2 °C, and decreased with body mass ($F_{1,120} = 13.347$, $p < 0.001$).

3.3. Metabolism and water loss

During RMR trials, frogs remained inactive with limbs and phalanges tucked under the body, head lowered and gular area in contact with the substrate. This posture has been described as a typical resting and water saving strategy in this species (Geise and Linsenmair, 1988). Resting MR was positively related to test temperature and body mass (Fig. 2a; Table 5; Table A2). In ANCOVA models, 15 °C-acclimated frogs had significantly lower RMR than 20 °C-acclimated frogs ($T = -2.325$, $p = 0.022$; Fig. 2a; Table A2). Orthogonal polynomial contrasts revealed a linear positive relationship between RMR and acclimation; a quadratic increasing relationship between RMR and test temperature, which had the strongest effect on RMR; and a non-significant interaction between acclimation and test temperature (Table 6). Overall, the results indicate partial support for 'colder is better' rather than the beneficial acclimation hypothesis (see predictions in Methods; Table 1).

Water loss rate did not vary significantly with acclimation or test temperature while frogs were at rest ($F_{2,96} = 0.923$ and $F_{2,96} = 0.498$ respectively; Fig. 2b; Table 5; Table A2). Body mass was the only significant predictor of resting WLR, and the ANCOVA model had limited explanatory power (Table A2). In orthogonal polynomial contrasts, neither acclimation, test temperature nor the interaction between them was significant (Table 6). Resting metabolic rate and resting WLR were correlated in only one of the nine treatment groups (15 °C-acclimated frogs tested at 20 °C: $rho = 0.664$, $p = 0.031$; all other $rho \leq 0.51$ and $p > 0.05$).

The best fit mixed model of AMR included significant effects of acclimation temperature, test temperature and body mass ($F_{2,26} = 17.989$, $p < 0.001$ (ACC); $F_{2,58} = 202.268$, $p < 0.001$ (TT); $F_{1,26} = 18.106$, $p < 0.001$ (body mass); Table 7). However, only test temperature

Table 4
Best-fit ANCOVA models for CT_{min} and CT_{max} using combined data from year 1 (2010/2011) and year 2 (2011/2012). ACC: acclimation temperature treatment (15, 20 or 25 °C). Bold text indicates significant parameters (alpha = 0.05). Model results for the separate years are shown in Table A1.

CT_{min} [a]				
Variable	DF	Type III SS	*F* value	*p* value
Intercept	**1**	**32.659**	**31.961**	**<0.001**
ACC	**2**	**16.950**	**8.294**	**<0.001**
Site	**2**	**13.842**	**6.773**	**0.002**
Sex	1	1.624	1.589	0.210
Year	**1**	**27.752**	**27.159**	**<0.001**
Body mass	**1**	0.328	0.321	0.572
ACC × body mass	**2**	**6.964**	**3.408**	**0.036**
Residuals	118	120.578		
CT_{max} [b]				
Variable	DF	Type III SS	*F* value	*p* value
Intercept	**1**	**19,095.900**	**4249.019**	**<0.001**
ACC	**2**	**52.300**	**5.813**	**0.004**
Site	2	16.500	1.831	0.165
Sex	1	0.500	0.113	0.738
Year	1	0.000	0.010	0.920
Body mass	**1**	**23.700**	**5.283**	**0.023**
ACC × year	**2**	**30.900**	**3.438**	**0.035**
Residuals	118	530.300		

Body mass was $\log_{10}$ transformed prior to analysis.
[a] Overall model multiple $R^2 = 0.4217$, $F_{9,118} = 9.561$, $p < 0.001$.
[b] Overall model multiple $R^2 = 0.4198$, $F_{9,118} = 9.485$, $p < 0.001$.

explained a substantial proportion of the deviance in the data (66% vs 5% (ACC) and 3% (body mass)), with higher temperatures resulting in higher metabolic rates (Fig. 2c; Table 7). AMR declined with acclimation to increased temperature, with significantly lower rates in 25 °C-acclimated frogs than in 15 °C- and 20 °C-acclimated frogs ($T = -3.893$, $p = 0.001$; Fig. 2c; Table 7). Active WLR did not change with acclimation, but increased strongly with test temperature, and was not affected by body mass (Fig. 2d; Table A3).

4. Discussion

4.1. Critical thermal limits and their plasticity

Critical thermal maximum extended above 40 °C in all acclimation groups. Although this is uncommon for anurans generally (Brattstrom, 1963, 1968; Rome et al., 1992), it is consistent with results for several other species of tropical and sub-tropical anurans (e.g. *Bufo marinus*, Snyder and Weathers, 1975) and earlier work on this species (Geise and Linsenmair, 1986). CT_{max} above 40 °C are also found in larvae of a range of sub-tropical and tropical amphibians (Duarte et al., 2012). Relatively high thermal tolerance may reflect painted reed frogs' adaptation to highly variable savanna habitats (Geise and Linsenmair, 1986; Schmuck and Linsenmair, 1988). Also, broad thermal tolerance is characteristic of species with large geographic ranges, wide latitudinal and altitudinal distributions (Brattstrom, 1968; Calosi et al., 2008; Gaston and Spicer, 2001).

Painted reed frogs tolerate a relatively wide range of temperatures, with mean critical temperatures per acclimation group ranging from 1.7 °C for CT_{min} to 45.9 °C for CT_{max}. As predicted, frogs acclimated at warmer temperatures had higher critical thermal limits than did those acclimated at cooler temperatures. The plasticity of CT_{max} may confer benefits on frogs from the novel range by increasing their survival in marginal thermal habitats. The higher acclimation response of upper than lower critical limits in this species is unusual among ectotherms, in which CT_{max} is usually more constrained than CT_{min} (Clusella-Trullas and Chown, 2014; Hoffmann et al., 2013; Snyder and Weathers, 1975; Sunday et al., 2011). However, the magnitude of plasticity of CT_{max} is in line with data for some widespread and restricted-range bufonid, hylid and ranid species in which CT_{max} can be adjusted by 4 to 6 °C in response to thermal acclimation (Brattstrom, 1968; Navas et al., 2008). Body mass was negatively related to CT_{max}. This relationship was however likely not due to experimental artefact in which a lag between the chamber temperature and the core body temperature leads to an overestimation of CT_{max} in larger animals. Pilot trials showed that core temperature equilibrated with ambient temperature within approx. 15 s (SJD, unpublished data), consistent with the very small body size of this species.

CT_{min} showed more complex temporal and spatial responses to acclimation than did CT_{max}: both year and site affected CT_{min}. Inter-annual variation in CT_{min} may be due to variation in environmental temperature patterns. Painted reed frogs reach reproductive maturity after one to two years (Bishop, 2004), so environmental temperatures in the years leading up to collections in December 2010 and December 2011 may account for differences in adult cold tolerance. However, this possibility is unlikely given that temperature data for the Cape Town area indicated that the spring leading up to the second collection was cool in relation to the long-term average. This should have resulted in lower and not higher CT_{min}. The spatial variation in CT_{min} among collection sites may be explained by existing genetic structure among populations in the novel range (Tolley et al., 2008). Rapid evolutionary adaptation of these traits cannot be excluded in the present study since Huey et al. (2003) and Logan et al. (2014) demonstrated that, in ectotherms, introduction to a novel thermal environment may result in directional selection of thermal performance traits in a relatively short time if sufficient genetic variation is present in the introduced population and traits meet the requirements for natural selection.

4.2. Response of metabolism to acclimation and test temperature

The lower RMR of frogs acclimated at the coldest temperature compared with those from the warmer treatments provides partial support for the 'colder is better' hypothesis, and suggests that these frogs can withstand seasonal cooling by minimising resting metabolism. This pattern is furthermore consistent with the compensation model of the fitness effects of basal metabolism (Boratyński and Koteja, 2010; Burton et al., 2011) which suggests that lowering resting metabolic rate allows extra resources to be allocated to activity by decreasing the baseline of aerobic metabolism (Killen et al., 2007). Indeed, the greatest difference between mass-adjusted resting and active CO_2 production in this study was in cold-acclimated frogs tested at the coldest temperature (see Table 5). However, this interpretation needs to be made with caution because the allocation of energy to other activities as a result of minimizing resting metabolism has not been demonstrated here.

Interpreting whether an acclimation response is beneficial or not is contingent on the ecology and behaviour of the organism being studied (Huey et al., 1999) and on the tissues or metabolic pathways involved in an acclimation response (Rogers et al., 2007). Here, the decrease in AMR with acclimation at the highest temperature (25 °C) can be interpreted as being advantageous, allowing frogs to sustain high levels of activity over the peak activity season in summer, when energetically-costly reproductive activities take place (e.g. chorus attendance and calling in males, and egg production in females). However, the allocation of energetic resources to these other activities has not been demonstrated in this study, and therefore the fitness benefits of reducing AMR at warm temperatures are difficult to discern. The down-regulation of AMR in warm-acclimated frogs could also indicate that physiological stress plays a role in the acclimation response at high temperatures. Although 25 °C is not likely a stressful temperature, constant exposure to this temperature over a long period may result in production of heat shock proteins (Bailey and Lazaridou-Dimitriadou, 1991; Dong et al., 2011; Marshall et al., 2011). The increase in AMR with test temperature

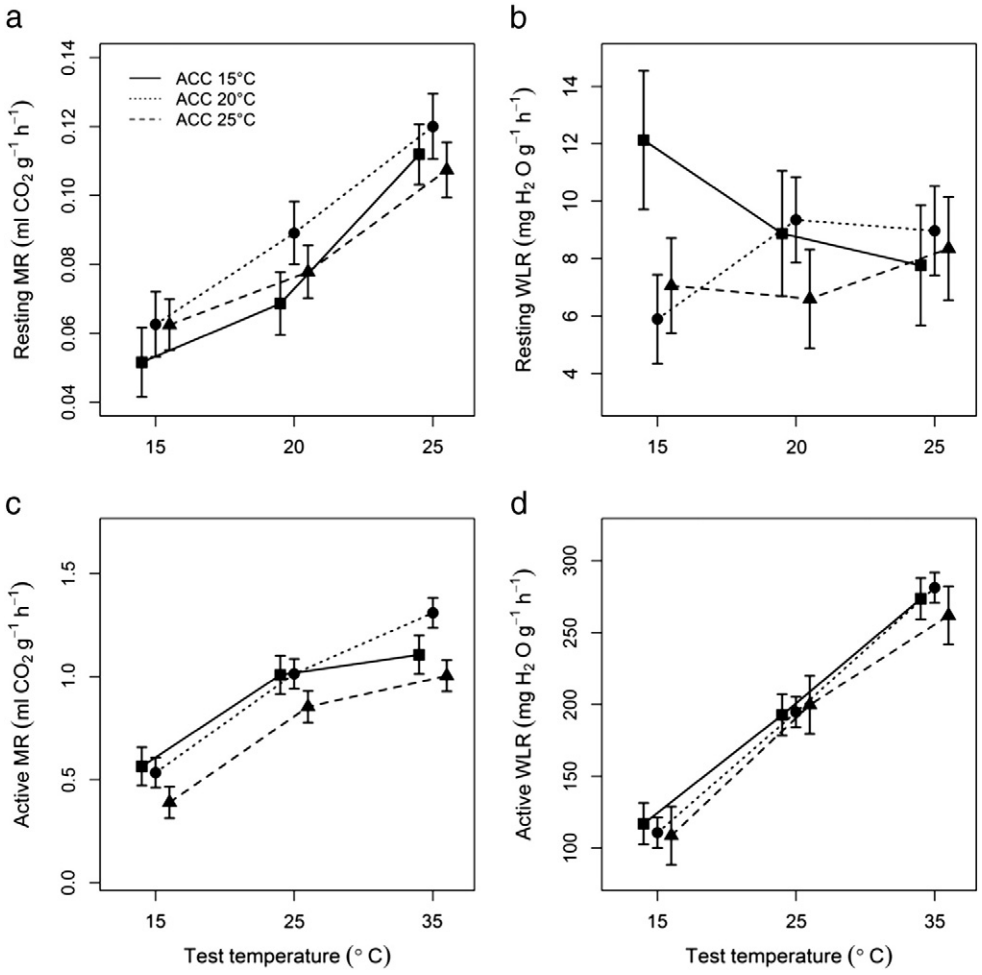

Fig. 2. Temperature dependence of active and resting metabolic and water loss rates of adult painted reed frogs acclimated at 15, 20 and 25 °C. Panels a and b show resting rates, panels c and d show active rates. Data are mass-adjusted (metabolic rate divided by body mass) and presented as means ± s.e.m. Note the different test temperatures used in resting (15, 20 and 25 °C) and active experiments (15, 25 and 35 °C).

Table 5
Body mass-adjusted metabolic and water loss rates of resting and active painted reed frogs. ACC: acclimation temperature treatment (15, 20 or 25 °C); TT: test temperature treatment (15, 20 or 25 °C for resting, and 15, 25 or 35 °C for active rates). MR and WL data are mean ± s.d. (alpha = 0.05).

Resting rates

ACC	TT	MR ($ml\ CO_2\ g^{-1}\ h^{-1}$)	WL ($mg\ H_2O\ g^{-1}\ h^{-1}$)	n
15	15	0.052 ± 0.010	12.131 ± 9.781	9
15	20	0.069 ± 0.014	8.875 ± 5.733	11
15	25	0.112 ± 0.022	7.767 ± 3.355	12
20	15	0.063 ± 0.013	5.894 ± 2.906	12
20	20	0.089 ± 0.030	9.352 ± 5.156	13
20	25	0.120 ± 0.021	8.973 ± 5.127	12
25	15	0.062 ± 0.018	7.060 ± 4.484	13
25	20	0.078 ± 0.011	6.596 ± 4.192	12
25	25	0.107 ± 0.017	8.351 ± 3.140	11

Active rates

ACC	TT	MR ($ml\ CO_2\ g^{-1}\ h^{-1}$)	x-fold difference (active:resting)	WL ($mg\ H_2O\ g^{-1}\ h^{-1}$)	x-fold difference (active:resting)	n
15	15	0.565 ± 0.101	11.0	116.933 ± 26.030	9.6	9
15	25	1.008 ± 0.153	9.0	192.698 ± 49.780	24.8	9
15	35	1.106 ± 0.101		273.686 ± 70.667		9
20	15	0.535 ± 0.091	8.5	110.686 ± 13.371	18.8	10
20	25	1.013 ± 0.118	8.4	194.672 ± 35.417	21.7	10
20	35	1.309 ± 0.083		281.304 ± 26.444		10
25	15	0.390 ± 0.041	6.2	108.597 ± 21.297	15.4	11
25	25	0.854 ± 0.144	7.9	199.711 ± 33.456	23.9	11
25	35	1.004 ± 0.329		262.030 ± 94.514		11

Table 6
Results of ordered factors ANOVA with orthogonal polynomial contrasts on resting metabolic and water loss rates of *Hyperolius marmoratus*. ACC: acclimation temperature treatment (15, 20 or 25 °C); TT: test temperature treatment (15, 20 or 25 °C). L denotes the linear contrast and Q the quadratic contrast. Bold text indicates significant parameters (alpha = 0.05).

Resting metabolic rate [a]

Variable	DF	Type III SS	*F* value	*p* value
Intercept	1	0.002	0.202	0.654
ACC	**2**	**0.089**	**4.898**	**0.009**
TT	**2**	**1.427**	**78.803**	**<0.001**
ACC × TT	4	0.039	1.065	0.378
Residuals	96	0.869		
	DF	Contrast SS	*F* value	*p* value
ACC (L)	**1**	**0.057**	**6.345**	**0.013**
ACC (Q)	1	0.003	0.373	0.543
TT (L)	1	0.014	1.564	0.214
TT (Q)	**1**	**1.405**	**155.162**	**<0.001**
ACC (L) × TT (L)	1	0.006	0.606	0.438
ACC (Q) × TT (L)	1	0.003	0.326	0.569
ACC (L) × TT (Q)	1	<0.001	0.029	0.865
ACC (Q) × TT (Q)	1	0.030	3.301	0.072

Resting water loss rate [b]

Variable	DF	Type III SS	*F* value	*p* value
Intercept	1	0.002	0.031	0.860
ACC	2	0.119	1.191	0.308
TT	2	0.066	0.666	0.516
ACC × TT	4	0.388	1.945	0.109
Residuals	96	4.784		
	DF	Contrast SS	*F* value	*p* value
ACC (L)	1	0.001	0.010	0.919
ACC (Q)	1	0.110	2.201	0.141
TT (L)	1	0.001	0.011	0.918
TT (Q)	1	0.081	1.616	0.207
ACC (L) × TT (L)	1	0.126	2.524	0.115
ACC (Q) × TT (L)	1	0.008	0.160	0.690
ACC (L) × TT (Q)	1	0.136	2.726	0.102
ACC (Q) × test (Q)	1	0.118	2.372	0.127

Rates were $\log_{10}$ transformed prior to analysis; contrasts were carried out using the residuals of the relevant rate-body mass relationship.
[a] Overall model multiple $R^2 = 0.108$, $F_{8,96} = 1.452$, $p = 0.185$.
[b] Overall model multiple $R^2 = 0.636$, $F_{8,96} = 20.96$, $p < 0.001$.

occurred over the full 15–35 °C range, suggesting that the activity of painted reed frogs is not limited by short-term exposure to high environmental temperatures.

Table 7
Results of best-fit linear mixed model fitted to active metabolic rate data of painted reed frogs. ACC: acclimation temperature treatment (15, 20 or 25 °C); TT: test temperature treatment (15, 25 or 35 °C). Bold text indicates significant parameters (alpha = 0.05).

Parameter	DF (num., den.)	*F* value	*p* value	% Deviance explained [a]
Intercept	**1, 58**	**7.553**	**<0.001**	
ACC	**2, 26**	**17.989**	**<0.001**	**5.485**
TT	**2, 58**	**202.268**	**<0.001**	**65.979**
Body mass	**1, 26**	**18.106**	**<0.001**	**2.760**
	Estimate	s.e.m.	*T* value	*p* value
Intercept	**−0.254**	**0.030**	**−8.477**	**<0.001**
ACC 20 °C	0.026	0.0267	0.975	0.339
ACC 25 °C	**−0.100**	**0.026**	**−3.893**	**0.001**
TT 25 °C	**0.293**	**0.019**	**15.355**	**<0.001**
TT 35 °C	**0.361**	**0.019**	**18.928**	**<0.001**
Body mass	**0.713**	**0.168**	**4.255**	**<0.001**

Body mass and metabolic rate were $\log_{10}$ transformed before analysis. Random effect: 1|indiv.
[a] Deviance explained is from the pamer.fnc function in LMERConvenienceFunctions package.

4.3. Lack of plasticity in water loss

Resting frogs maintained a water-conserving posture and had very low rates of evaporative water loss (5.9 mg g^{-1} h^{-1} to 12.1 mg g^{-1} h^{-1}) when measured in dry air. These levels are consistent with the high cutaneous resistance (*R*) known for this species and its close relatives (Geise and Linsenmair, 1988; Withers et al., 1982). The mechanism behind the high *R* in this species is unknown, but may be related with skin structure, such as the presence of large numbers of iridophores and low vascularisation, but not to skin secretions as in some other anuran species (Withers et al., 1982, c.f. Gomez et al., 2006; Shoemaker et al., 1989). The lack of response of resting WLR to test temperature and the sizeable change in WLR between resting and active frogs illustrate the profound effect that behaviour, specifically body posture, has on water loss in this species.

Since maximum environmental temperatures are experienced during the day, frogs need to seek optimal resting microsites when water loss associated with activity is at its highest. Reliance on a particular body posture to minimise water loss therefore limits mobility and may prevent frogs from regulating body temperature precisely (see Buttemer, 1990; Tracy, 1976; Tracy and Christian, 2005). The fact that neither resting nor active WLR responded to acclimation suggests that the water-conserving posture may inhibit selection for phenotypic plasticity in water loss in this species, reflecting behavioural inertia (Huey et al., 2003; Pichegru et al., 2010). However, this scenario needs further testing by estimating if the costs associated with maintaining the water-conserving posture balance the benefits under most conditions.

In addition to water loss, overall water balance in amphibians is a consequence of water uptake and stored body water (e.g. bladder stores). Modification of water uptake rates could be a more effective route to adjusting water balance than variation in water loss in this species, as water uptake is controlled by the endocrine system through relatively simple hormonal feedbacks in response to environmental conditions (Hillman et al., 2009). Water uptake through the pelvic patch has been shown to occur rapidly in painted reed frogs (69% of initial body mass over 30 min; Geise and Linsenmair, 1986, 1988). However, frogs need to be inactive with the ventral surface in contact with a moist substrate to take up water, and therefore direct benefits from plasticity of water uptake via this mode would not be available when frogs move or thermoregulate.

Because of the relatively impermeable dorsal surface of *H. marmoratus* (i.e. high cutaneous resistance, $R > 500$ s cm^{-1}; Withers et al., 1982), these highly desiccation-resistant frogs cannot necessarily use evaporative cooling effectively, and may have body temperatures that are equal to or elevated above ambient (Passmore and Malherbe, 1985). This is particularly likely to be true of small anuran species which are confined within the boundary layer of the substrate (Tracy et al., 2010). Under conditions of heat stress, these frogs may make use of evaporative cooling by releasing mucus from skin glands (Geise and Linsenmair, 1986; Tracy et al, 2008). The presence of this mechanism suggests that a trade-off could exist between evaporative cooling as a thermoregulatory strategy and evolving a high *R* to minimise water loss. In other words, the lack of plasticity of water loss, combined with the reed frogs' known high *R*, suggests that desiccation resistance may have evolved at the expense of plasticity in *R*, and that the reduction of *R* that would be needed to aid evaporative cooling as a thermoregulatory strategy did not evolve in this species. This may be attributed partly to the high availability of optimal thermal habitat within the species' native range, and underscores that absolute environmental tolerance may be traded-off against plasticity of tolerance. Such a possibility is not unreasonable, as for example, in ectotherm heat tolerance, trade-offs between elevated basal tolerance and high plasticity of tolerance have been found in interspecific comparisons (e.g. Stillman, 2003).

Neither resting nor active *H. marmoratus* showed the sharp or non-linear increase in WLR at high temperatures that is observed in some

water-resistant frogs, such as Australian *Litoria bicolor* and *L. xanthomera* (Tracy et al., 2008). However, evaporative cooling at temperatures close to CT_{max} was recorded in West African *H. viridflavus nitidulus* (=*H. nitidulus*) (Geise and Linsenmair, 1986), a species closely related to *H. marmoratus*. It is possible that we did not observe these changes in our study animals simply because they were exposed to benign conditions or that factors other than temperature may drive plasticity of water loss rate. A longer temperature regime (e.g. a seasonal cycle) may be needed to elicit the physiological changes necessary to modify water loss rate through, for example, changes in membrane structure. Geise and Linsenmair (1986) found that *H. viridflavus nitidulus* collected from West African savannas during the dry season ('dry season frogs') had exceptionally low water loss rates while 'wet season frogs' had water loss rates similar to those of non-desiccation resistant amphibian species, suggesting that a seasonal or age-related adjustment of water loss rates exists. The water loss rates found in our study of South African frogs were consistent with the dry season pattern described by Geise and Linsenmair (1986)—maintaining low WLR with no plasticity thereof, and relying on their posture and skin properties to minimise water loss. It is possible that the close coupling between desiccation resistance and thermoregulation (Navas et al., 2008; Tracy et al., 2008, 2010) has dampened the development of plasticity of WLR in painted reed frogs. However, the reasons for the lack of plasticity in water loss need further investigation in these and other 'waterproof' frogs.

5. Conclusion

Our examination of the responses of key physiological traits to thermal acclimation in *H. marmoratus* demonstrates substantial plasticity in thermal tolerance (particularly the upper thermal limit), resting and active metabolism, but not in water loss rate at rest or during sub-maximal activity. The lack of plasticity in water loss rate over a temperature range of 15 °C to 35 °C likely limits dispersal of *H. marmoratus* in the dry summer reproductive season, while the ability to maintain activity during cooler periods may allow increased dispersal in winter. These physiological characteristics have the potential to influence this species' current and future dispersal and inter-pond movement patterns at least in summer within the novel range.

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Appendix A. Supplementary data

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